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Screening of Bacteriocin Producing Lactic Acid Bacteria for Probiotic Properties

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ABSTRACT
The concept of providing functional foods including beneficial probiotic components is gaining attention in recent years. Therefore, this study was aimed to isolate potential probiotic Lactic Acid Bacteria (LAB) from various sources. A total of 66 LAB strains were selectively isolated from different sources using MRS agar medium. Out of 66 isolates, only 7 were found to be bacteriocin producers and showed antibacterial activity against *S. aureus* and *E. coli*. All the 7 isolates were screened for probiotic properties like bile tolerance (1000 to 10,000 ppm bile salt concentration), acidic pH tolerance (pH 1 to 3.5), auto aggregation potential and cell surface hydrophobicity. Isolates RM4 & RM7 were able to tolerate bile salt concentration of up to 10,000 ppm. For acidic pH tolerance, both the isolates RM4 & RM7 were able to grow at pH 3.5 to 2. The autoaggregation potential and cell surface hydrophobicity of RM7 was found to be 40.88% and 54.58% respectively but these properties were found to be below 40 % for RM4. Therefore isolate RM7 fulfilled the criterion to be identified as a possible potential probiotic strain.

Key words: Lactic acid bacteria, Probiotics, Bile tolerance, Hydrophobicity, Autoaggregation

INTRODUCTION

A plethora of studies are in progress to evaluate and improve the health benefits attributed to Lactic Acid Bacteria (LAB) because of the century old hypothesis that some specific dairy products fermented by LAB may provide health benefits [1]. LAB are Gram-positive, catalase negative, anaerobic but aero-tolerant, non spore forming rods or cocci that produce lactic acid as the major end product from the fermentation of carbohydrates [2]. LAB are commonly known to produce antimicrobial substances such as bacteriocins in foods, thereby possessing a great potential to be used as food biopreservatives [3].The antimicrobial potential of lactic acid bacteria has been appreciated for more than 10,000 years and has enabled to extend the shelf life of many foods [4].

Nowadays, people are aware that diet plays a major role in preventing diseases and promoting health. Therefore there is an increasing trend for foods containing probiotic cultures [5]. Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the host [6]. Some LAB positively influence human health mainly by improving the composition of intestinal micro biota and for this reason, they are called probiotics [7]. The increasing cost of health care, the steady increase in life expectancy and the desire of the elderly for improved quality of their lives are driving factors for research and development in the area of probiotics and also the concept of providing functional foods including beneficial components rather than removing potentially harmful components is gaining attention [8].

MATERIALS AND METHODS

Sample collection and processing

Fifty samples were collected for isolation of Lactic acid bacteria. Out of 50 samples, 20 samples were of Homemade dahi/curd (HM), 14 samples of Raw milk (RM) collected from different milk vendors, 10 samples were of Whey (W) collected from nearby dairy and sweet shops, 4 samples of packaged milk of Verka brand and Reliance brand, 1 sample was of Uttam dahi/curd and 1 was of Verka dahi/curd. After sample collection, processing of the samples was carried out according to the method given by Sharma *et al.* [9].

Isolation of lactic acid bacteria

The selective isolation of LAB was carried out using MRS medium. For selective isolation, 1ml of sample was homogenized with 9 ml of 0.85% sterile saline and then serially diluted. 0.1 ml of the diluted sample was inoculated on MRS agar plates. The plates were incubated at 37°C for 24 hrs [10]. Isolated colonies were maintained on MRS agar slants at 4°C and periodic revival of isolates was done after every 15 days. Screening tests were carried out on each isolate. A "selection by rejection technique" was used to select likely cultures i.e. only isolates that met the criteria of first test (i.e. bacteriocin production test) was selected for next test for the probiotic properties viz. bile tolerance test, acid tolerance test, auto aggregation assay and hydrophobicity assay [11].

Screening of isolates for bacteriocin production

The cultures of *Escherichia coli* and *Staphylococcus aureus* were grown in nutrient broth for 24 h. 0.5 Mc Farland Standard was prepared to have the cell concentration of $1x10^8$ at wavelength 600 nm. To prepare 0.5 Mc Farland Standard, 995 ml of 1 % sulphuric acid was mixed with 5 ml of 1% barium chloride and a turbid solution was obtained due to formation of barium sulphate precipitate.

Agar well diffusion assay procedure was used for screening of isolates for bacteriocin production [10].

For this, Mueller-Hinton (MH) agar plates were prepared and 0.1 ml cultures of *E. coli* and *S. aureus* which were prepared in accordance with 0.5 Mc Farland standards were spreaded on MH agar plates. The plates were incubated for 20 min at 37 °C. Wells on MH agar plates were made with sterilized borer. The MRS broth culture supernatant of each isolate was obtained by centrifuging the culture and the pH of the supernatant fluids was then adjusted to pH 6.5- 7 with 5N NaOH or 5N HCl to rule out any inhibition by the production of organic acids. Wells were then filled with neutralized supernatant (50 – 100 µl). The plates were then incubated at 37°C for 24 hrs. After incubation, plates were observed for inhibition zones.

Characterization of isolates

The selected isolates were characterized by morphological, physiological and biochemical methods [3]. Morphological characterization was done by colony morphology and gram staining while physiological characterization by growing at different temperatures (10, 20, 37 and 45°C), different pH (5, 6, 8 and 9) and varying salt concentrations (5, 10 and 15%). Biochemical tests like catalase test, oxidase test, NH_3 from arginine, glucose fermentation test, nitrate reduction test, carbohydrate utilization tests and gelatine hydrolysis test were carried out.

Evaluation of probiotic properties

a) Bile tolerance test: To test the bile tolerance of isolates, the isolates were grown at 37°C without bile salt and then plated on MRS agar with different bile salt concentrations. Incubation was done at 37°C for 24-48 hrs. Different Bile salt concentrations used for the test were 1000 to 10,000 ppm. MRS agar with no bile salt was used as control [12].

b) Acidic pH tolerance test: For this test, the isolates were grown in MRS broth with pH 2, 2.5, 3 and 3.5. Incubation was done at 37 °C for 24 – 48 h. MRS broth with pH 6 was used as control [12].

c) Auto-aggregation assay: The test strains were grown in 3 ml of MRS broth of pH 6 with cysteine and cells were harvested at 2,400 x g. The pellet of cells was washed twice with PBS (0.02M and pH 7.4). The cells were re-suspended in PBS to an OD of 0.5 units at 600 nm. 3ml from this suspension was taken and cells were harvested at 2,400 x g. The harvested cells were re-suspended in their original broth and incubated at 37°C for 2 hrs. 1 ml of culture was taken from the upper portion of the culture and OD was measured. Finally, culture was shaken and total OD was measured [13].

Auto aggregation (%A) = 1 - [OD of upper portion\ OD total] x 100

d) Cell surface hydrophobicity: For this test, the strains were grown in 3 ml MRS broth of pH 6 with cysteine and cells were harvested at 2,400 x g. The pellet of cells was washed twice with PBS (0.02M and pH 7.4). The cells were re-suspended in PBS and 2 ml suspension was transferred in another test tube. 0.4ml xylene was added and tubes were shaken for 2 min and reposed for 15 min. OD of the aqueous phase was measured at 600nm. The decrease in OD of aqueous phase was considered as a measurement of cells surface hydrophobicity (%H) [13].

Hydrophobicity (%H) = $[(A_0 – A)/A_0] \times 100$

Where,

A_0 = Absorbance before xylene extraction

A = Absorbance after xylene extraction

RESULTS AND DISCUSSION

A total of 66 strains were isolated from various samples. The strains were preserved on MRS agar slants and used for further screening.

Screening of Isolates for Bacteriocin Production

All the 66 isolates were tested for bacteriocin production by agar well diffusion assay method. Out of all the isolates, only 7 isolates were found to be bacteriocin producers (Table 1). The isolates were designated as RM4, RM7, HM2, HM7, HM18, W6, W7 based upon their source. Maximum number of bacteriocin producers was isolated from samples of homemade dahi (HM2, HM7 and HM18). Only 2 isolates from whey (W6 and W7) and 2 from raw milk (RM4 and RM7) were found to be bacteriocin producers. The antibacterial activity was tested against *Escherichia coli* and *Staphylococcus aureus*. Zone of inhibition various isolates is shown in Figure 1. Isolate HM18 showed inhibitory activity against only *Escherichia coli* while isolates W7, HM2, HM7, W6, RM4 and RM4 showed activity against *Staphylococcus aureus* only. Therefore, the present study confirms the occurrence of bacteriocin producing lactic acid bacteria in milk products as reported by other investigators [14] and it is also clear from this study that gram positive indicator bacterium was much more sensitive to bacteriocin of isolates than gram negative indicator bacterium. This has also been reported that the gram positive bacteria are most sensitive to bacteriocin of LAB than gram negative bacteria due to the particular nature of their cellular envelopes and bacteriocin act by adsorption to the cells [16]. Thus, the inhibitory effect of bacteriocin of lactic acid bacteria varies among Gram positive and negative organisms.

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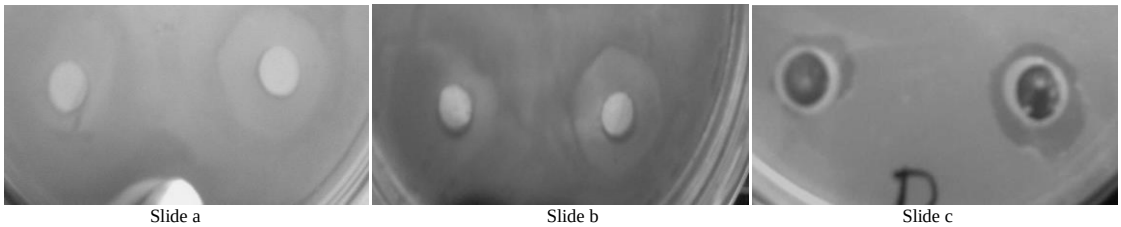
Table1. Bacteriocin producing lactic acid bacteria from different products

Samples	Tested isolates	Positive isolates	Designation	Antimicrobial activity		
				S. aureus	*E. coli*	Zone of Inhibition (mm)
Raw milk	22	2	RM4	+	-	11
			RM7	+	-	16
Packaged Milk	2	0	-			
Homemade Dahi	27	3	HM2	+	-	4.0
			HM7	+	-	10
			HM18	+	-	6.0
Packaged Dahi	3	0	-			
Whey	12	2	W6	+	-	4.0
			W7	+	-	9.0
+: Zone of Inhibition Observed, -: No Zone of Inhibition Observed						



Slide a Slide b Slide c

Fig. 1 Zone of Inhibition of RM4 & RM7 (slide a) HM2 & HM7 (slide b) W6 & W7 isolates (slide c) against *Staphylococcus aureus*

Table 2. Characterization of isolates

Isolates	RM 4	RM 7	HM 2	HM 7	W6	W 7	HM 18
Colour Colony Morphology	White coloured, smooth, opaque colonies with round margin	Whitish, smooth, glistening colonies with round margin	Cream coloured, pin point, smooth, circular colonies	White coloured, smooth, opaque colonies with round margins	Small white coloured colonies with round margins	Large white coloured, circular and opaque colonies	White coloured, smooth, opaque colonies with round margins
Gram Staining	Gram positive rods	Gram positive rods	Gram positive rods	Gram positive cocci	Gram positive cocci	Gram positive cocci	Gram positive cocci
Catalase Test	-	-	-	-	-	-	-
Nitrate Reduction Test	-	-	-	-	-	-	-
NH_3 from Arginine	-	-	-	+	+	+	+
CO_2 from glucose	-	-	-	-	-	-	-
Oxidase Test	-	-	-	-	-	-	-
Galactose utilization	+	+	+	+	+	+	+
Lactose utilization	+	+	+	+*	+*	+*	+*
Sucrose utilization	+	+	+	+*	+	+	+*
Maltose utilization	+	+	+	+	+	+	+
Gelatin Hydrolysis	-	-	-	-	+	+	-
+: Positive result, -: Negative result, +*: Delayed fermentation							

Table 3. Growth of isolates at different temperatures, pH and salt concentrations

Isolates	Temperature				pH				Salt Concentration		
	10ºC	20ºC	37ºC	45ºC	5	6	8	9	5%	10%	15%
RM4	LG	MG	GG	GG	MG	GG	GG	GG	GG	MG	NG
RM7	LG	MG	GG	MG	MG	GG	GG	GG	GG	MG	NG
HM2	LG	MG	GG	NG	LG	MG	GG	GG	LG	NG	NG
HM7	LG	MG	GG	GG	MG	GG	GG	GG	NG	NG	NG
HM18	LG	GG	GG	NG	NG	NG	GG	MG	NG	NG	NG
W6	MG	GG	GG	MG	LG	MG	GG	GG	NG	NG	NG
W7	LG	GG	GG	GG	NG	NG	GG	GG	LG	NG	NG
NG: no growth, **LG:** light growth, **MG:** moderate growth and **GG:** good growth											

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Table 4. Growth of isolates in different bile salt concentration

Bile Salt Conc. (ppm)	Isolates						
	RM4	RM7	HM2	HM7	HM18	W6	W7
1000	+	+	+	+	+	+	+
2000	+	+	+	-	-	-	-
3000	+	+	-	-	-	-	-
4000	+	+	-	-	-	-	-
5000	+	+	-	-	-	-	-
10,000	+	+	-	-	-	-	-
+ : Growth, - : No Growth							

Table 5 . Growth of isolates at acidic pH values

pH	Isolates						
	RM4	RM7	HM2	HM7	HM18	W6	W7
3.5	+	+	+	+	-	-	+
3.0	+	+	-	-	-	-	-
2.5	+	+	-	-	-	-	-
2.0	+	+	-	-	-	-	-
1.0	-	-	-	-	-	-	-
+ : Growth, - : No Growth							

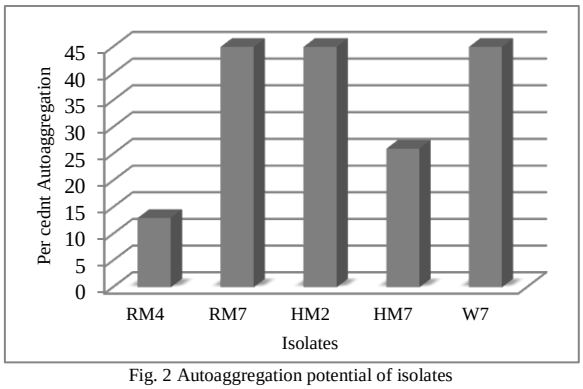

Fig. 2 Autoaggregation potential of isolates

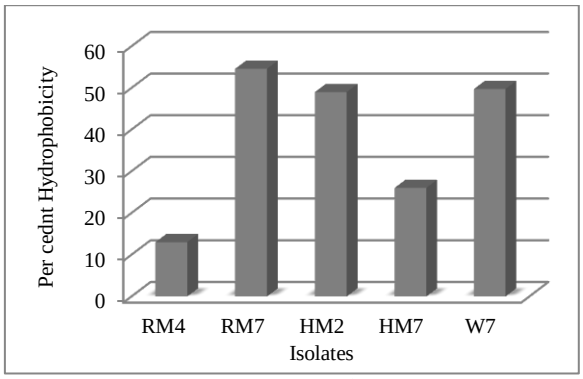

Fig. 3 Hydrophobicity of isolates

Characterization of Isolates

All the selected isolates were further characterized morphologically, biochemically and physiologically. The colony morphology of all 7 isolates was observed by streaking over MRS agar. Colonies formed by most of the isolates were white to cream

in colour with smooth and round margins (Table 2). Isolate RM7 showed glistening colonies. Gram staining was also performed to find out cell morphology and gram reaction of the isolates. All the isolates were gram positive. Similarly, LAB isolated from cheese, which were found to be gram positive and produced small round or lenticular white colonies on MRS agar [15]. It has also been reported that lactic acid bacteria are gram positive and produce white creamy colonies on MRS agar [13].

All the isolates were found to be negative for catalase and nitrate reduction test. Results of other tests used for the characterization of isolates are shown in Table 2.

For physiological characterization, growth of isolates at different temperature, pH and salt concentrations was observed. The results are shown in Table 3. All the isolates were observed to grow at 10ºC, 20ºC & 37ºC but at 45ºC, only HM2 and HM18 were able to grow. However, growth at temperatures below 37ºC was not as good as at 37ºC for all the isolates. Similar results have been reported that lactic acid bacteria grow luxuriously at 37ºC and weakly at 10ºC [3].

The optimum pH for growth of all the isolates was found to be 8 and 9 with no growth of HM 18 and W7 at pH 5 and 6 while all other isolates were able to grow at these pH values. In case of salt (NaCl) concentration, 15 % NaCl concentration was found to be inhibitory for all the isolates, only RM4 and RM7 were able to grow moderately at 10% while at 5% salt concentration HM2 and W7 were able to grow in addition to RM4 and RM7. Present results are in accordance with other studies that lactic acid bacteria grow best at 6.5% NaCl and at pH 9.6 [15].

Evaluation of probiotic properties of isolates

The performed experiments aimed at evaluating the properties viz. resistance to bile salts, survivability in the environment with different pH, auto aggregation potential & cell surface hydrophobicity of all 7 strains of potentially probiotic bacteria.

Bile tolerance is known to be one of the essential properties required for LAB to survive in the small intestine and it plays important role in physiological functions [16]. Bile salt concentrations used for this test were 1000 to 10,000 ppm. Among the tested isolates, the bile tolerance capacity of only RM7 and RM4 was upto10,000 ppm (Table 4). Therefore, the bile resistance of RM4 and RM7 was higher than HM2, HM7, HM18 and W6, W7. Similarly, it has been reported that *Lactobacillus* strains isolated from cheese were able to survive at bile salt concentration of 10,000, 15,000 and 20,000ppm after 48hr of incubation at 37ºC [17]. In the human gastrointestinal tract, the mean bile salt concentration is believed to be 3000ppm, which is considered as critical and high enough to screen for resistant strain [18].

For the capability of the selected strains to survive in conditions of the gastrointestinal tract, growth at variable acidic pH is important. Acid tolerance is a fundamental property that indicates the ability of probiotics to survive passage through the stomach [12]. Survival of all isolates was examined at pH between 1 and 3.5 (Table 5). Isolates RM4, RM7, HM2, HM7 and W7 were found to be resistant to pH 3.5 while HM18 and W6 were not able to grow at this pH. Only two isolates, RM7 and RM4 were able to resist pH up to 2. It has been reported that some *Lactobacillus* strains are able to retain their viability even at pH 1 [19]. In general RM4, RM7, HM2, HM7 and W7 showed a good resistance to low pH. Therefore, it has been assumed that these isolates may survive passage through the digestive system that has specific condition such as the low pH of the stomach. Hence, RM7, RM4, HM2, HM7 and W7 were selected for further tests as these were found to be resistant to pH 3.5.

Another desirable property of probiotic bacteria is the colonization in intestinal wall. This colonization is necessary in order to exert its beneficial effects [20]. It is important to evaluate surface properties, like auto-aggregation and hydrophobicity, because they are used as a measurement directly related to adhesion ability to enterocytic cellular lines [21]. Autoaggregation determines the capacity of the bacterial strain to interact with itself in a nonspecific way. And when hydrophobicity is high (more than 40%), it indicates the presence of hydrophobic molecules in the bacterial surface, like surface array proteins; wall intercalated proteins, cytoplasmic membrane protein and lipids [22]. Five isolates RM7, RM4, HM2, HM7 and W7 were selected for autoaggregation and hydrophobicity assay. Isolates showing autoaggregation percentage above 40% indicates that the isolates can be considered to have probiotic effects which related to adhesion to epithelia [13]. Isolates RM7 and HM7 showed autoaggregation potential of 40.88 % and 43 % respectively while the autoaggregation capacity of all other isolates was below 40% (Figure 2).

In case of hydrophobicity, RM7 showed hydrophobicity of 54.58 %, W7 showed 49.72 % and HM2 showed 48.99 %. Hydrophobicity of all other isolates was below 40% (Figure 3). This indicates that isolates RM7, W7 and HM2 have hydrophobic characteristics. From these results it is clear that only isolate RM7 can be considered as probiotic because the autoaggregation as well as hydrophobicity of this isolate was higher than 40 % which is the minimum necessity for considering a strain to be probiotic [21, 23]. Furthermore, the bile tolerance and acidic pH tolerance capacity of RM7 was also good. Therefore, RM7 fulfilled the criteria to be considered as potential probiotic strain.

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